Psoriasin (S100A7) is a major *Escherichia coli*-cidal factor of the female genital tract

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The female urogenital tract requires an efficient defense against bacteria, potentially derived from the adjacent intestinal tract. We have thus sought to identify the factors that protect against *Escherichia coli* (*E. coli*) in the female genital tract. Vaginal fluid from healthy human donors consistently killed *E. coli* *in vitro* and vaginal epithelium strongly expressed and secreted psoriasin. Psoriasin was constitutively produced in an organotypic vaginal epithelium model, and exposure of these cells to supernatants of *E. coli* cultures led to an enhanced psoriasin expression. Secreted psoriasin in vaginal fluids accounted for approximately 2.5–3% of total protein. Fractionation of vaginal fluids by high performance liquid chromatography (HPLC) showed that psoriasin co-eluted with a peak of *E. coli* killing activity. Our data show that normal vaginal fluid contains a powerful intrinsic antimicrobial defense against *E. coli* and that psoriasin contributes to the innate immune response of the female genital tract.

INTRODUCTION

The female genital tract is a complex biosystem constantly exposed to a multitude of bacterial, fungal, and viral agents. A fine balance between growth control of microorganisms of the normal vaginal flora and a rapid elimination of pathogens is crucial for the maintenance of epithelial integrity and function. The epithelial cells of the vaginal mucosa represent the first line of defense against infections. They secrete cytokines and chemokines that recruit cells of the immune system to sites of infection, and themselves attack potential pathogens by releasing antimicrobial peptides (AMPs).^{1–3} AMPs with activities against Gram-positive as well as Gram-negative bacteria, fungi, and viruses^{4–6} have been found to be produced by vaginal epithelium and include α -defensin-5, β -defensins 1 through 5, lysozyme, cathelicidin, and secretory leukocyte protease inhibitor.^{7–10}

Escherichia coli is present in large quantities in the lower gastrointestinal tract and is therefore anatomically close to the female urogenital tract. Rapid elimination of this pathogen would thus be important to prevent *E. coli* infections in this area. Psoriasin (S100A7), a member of the S100 family of proteins,^{11,12} is produced by epidermal keratinocytes¹³ and has recently been identified as an AMP with strong activity against *E. coli*.^{13,14}

Although present in normal epidermis, in which it is focally expressed at locations with high bacterial load,¹³ its expression can be strongly stimulated *in vivo* by exposure of healthy human skin, or *in vitro*, of cultured keratinocytes to supernatants of *E. coli*.^{13,15} Molecular analyses identified the bacterial surface component flagellin as the main inducer of psoriasin.¹⁵

We hypothesized that the female urogenital tract could be protected from *E. coli* infection by release of *E. coli*-cidal AMPs, and have investigated the expression and regulation of psoriasin in the lower female genital tract. We show that this AMP is highly expressed by stratified squamous vaginal epithelium, but not by cylindrical cervical mucosa, and that a high amount of psoriasin is present in normal vaginal fluids. In addition, we could show that vaginal fluids show strong *E. coli*-cidal activities. Our study identifies psoriasin as an important component of the innate immune system of the female genital tract.

RESULTS

Vaginal fluid shows strong *E. coli*-cidal activity

To analyze whether vaginal fluids are able to kill *E. coli*, we incubated bacterial suspensions with 20% of vaginal lavages for 3 h and then plated the bacteria on LB-agar. All vaginal lavages tested ($n = 8$) showed strong *E. coli*-cidal activities (**Figure 1a**).

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Received 21 January 2010; accepted 26 May 2010; published online 23 June 2010. doi:10.1038/mi.2010.37

Figure 1 Vaginal fluids show strong antimicrobial activity against *Escherichia coli*. (a) Vaginal fluids were tested for their *E. coli*-cidal activity in the microdilution assay system. *E. coli* was incubated with 20% of vaginal lavages for 3 h. After plating and incubation overnight at 37 °C, colonies were counted and the percentage of killing was calculated. (b) Vaginal lavages at concentrations between 1 and 20% were tested for their *E. coli*-cidal activity in the microdilution assay system; 1% showed 25% killing activity, and 100% killing was detected using a concentration of 5%. Error bars represent 1 s.d. calculated from three vaginal fluids.

Indeed, even a 1% solution of the vaginal lavages showed antimicrobial activity and a concentration of 5% was sufficient to completely eliminate this pathogen (Figure 1b).

Psoriasin is highly expressed in the female genital tract *in vivo* and is induced by *E. coli* in a three-dimensional organotypic vaginal epithelial model *in vitro*

As psoriasin is the major *E. coli*-killing factor in the skin,¹³ we investigated whether this AMP is present in the lower female genital tract and whether it contributes to the *E. coli*-cidal activities detected in vaginal lavages. When we analyzed biopsy specimens from vagina ($n = 10$), vulva ($n = 5$), and cervix ($n = 10$) by immunohistochemistry, we found strong expression of psoriasin in epithelia of the vulva (Figure 2b), the vagina (Figure 2c), as well as the ectocervical part of the cervix (Figure 2d). As shown in Figure 2d, psoriasin expression was present in the ectocervix (Figure 2d, right part) and absent beginning from the transition to squamous metaplasia (Figure 2d, left part). In contrast, psoriasin was only focally expressed in normal skin (Figure 2a), confirming earlier reports by others.¹³ Virtually no expression of psoriasin was found in endocervical epithelium (Figure 2e).

A three-dimensional organotypic vaginal epithelial model was used to investigate whether psoriasin expression *in vitro* is constitutive and/or induced by bacterial components. Using immunohistochemistry (Figure 3a, b) and western blot analysis (Figure 3c) we found constitutive expression of psoriasin in samples of untreated organotypic vaginal epithelium (Figure 3a) and upregulation of psoriasin after incubation of the organotypic vaginal epithelial model with heat-inactivated *E. coli* supernatants (Figure 3b). Enzyme-linked immunosorbent assay (ELISA) showed that up to 10 ng ml⁻¹ psoriasin was secreted in the culture medium of untreated organotypic vaginal epithelium (Figure 3d). The addition of heat-inactivated *E. coli* supernatants led to a 6.6-fold increase in psoriasin secretion (Figure 3d). In contrast, treatment with culture supernatants of *Lactobacillus acidophilus*, which is a part of the physiological vaginal bacterial flora, had no influence on psoriasin expression (data not shown).

To quantify the amount of psoriasin in vaginal fluids, we rinsed vaginas of healthy volunteers with 2 ml physiological sodium chloride solution and analyzed the filtered lavages by ELISA (Figure 4a) and western blotting (Figure 4b). Lavages from four different volunteers contained psoriasin concentrations of up to 1,850 ng ml⁻¹, with a mean value of 1,370 (± 550) ng ml⁻¹ (Figure 4a). In addition, western blot analysis of 3 μ g of the same lavages showed a much stronger specific immunoreaction than 30 μ g of stratum corneum extracts of healthy skin (Figure 4b).

To compare the amount of psoriasin with the whole protein spectrum of vaginal lavages, we performed two-dimensional electrophoresis followed by western blot analysis. Whole protein staining of the nitro-cellulose membrane with deep-purple led to the detection of approximately 30 major proteins (Figure 4c, left panel). Incubation of the same membrane with an anti-psoriasin monoclonal antibody revealed a single specifically reacting spot (Figure 4c, right panel). Psoriasin was detected at a molecular mass of 11.3 kDa and an isoelectric point of 5.6 (Figure 4c). Analysis with ImageQuant TL (GE Healthcare, Vienna, Austria) and Melanie software (Genebio, Geneva, Switzerland) showed that psoriasin accounts for approximately 2.5–3.0% of the whole protein content of vaginal lavages.

Psoriasin is an *E. coli*-cidal protein of vaginal fluid

To further investigate the role of psoriasin in the *E. coli*-cidal capacity of the vagina, we fractionated vaginal fluids by reversed-phase high performance liquid chromatography (HPLC). A representative analysis of a psoriasin-rich vaginal fluid is shown in Figure 5a.

Aliquots of each HPLC fraction were subjected to a radial diffusion antimicrobial assay with *E. coli* as microbial target. We mostly detected three peaks of *E. coli*-cidal activity (Figure 5a). Electrospray ionization mass spectrometry (ESI-MS) analyses revealed in *E. coli*-cidal activity-containing HPLC fraction masses of 3,446, 14,692, and 11,366 Da, which correspond exactly to the α -defensin human neutrophil peptide-1 (HNP-1), lysozyme, and a N-acetylated form of psoriasin, which lacks the aminoterminal methionine.¹³ To confirm psoriasin in the late-eluting antimicrobial

Figure 2 Psoriasin is expressed in vulva, vaginal, and ectocervical epithelium but not in endocervical epithelium. To localize psoriasin expression in the female genital tract immunohistochemistry was performed using a mouse monoclonal antibody against human psoriasin. In contrast to normal human skin (**a**), the expression of psoriasin in (**b**) the vulva, (**c**) the vagina, and (**d**) ectocervical epithelium was much stronger and persistent through several suprabasal layers. (**d**) A transition site from a squamous metaplasia (left part) to ectocervical epithelium (right part) is shown. Note that only the ectocervical part shows strong psoriasin expression. No expression of psoriasin was found in the endocervical epithelium (**e**). (**f**) The same vaginal sample as shown in **c** was incubated with normal mouse IgG₁ as isotype control followed by the appropriate secondary antibody.

Figure 3 Psoriasin is constitutively expressed in an *in vitro* reconstructed three-dimensional (3D) organotypic vaginal epithelial model and is upregulated by exposure to heat-inactivated *Escherichia coli*. An *in vitro* reconstructed 3D organotypic vaginal epithelium was cultured under (**a**) normal conditions or (**b**) exposed for 24 h to heat-inactivated *E. coli* and analyzed for psoriasin expression by (**a**, **b**) immunohistochemistry, (**c**) western blot analysis, and (**d**) enzyme-linked immunosorbent assay (ELISA). The samples in **c** were normalized to GAPDH expression. Expression of psoriasin in cell lysates was increased 3.5-fold and secretion of psoriasin into the culture medium was increased 6.6-fold after treatment with *E. coli* culture supernatants. This experiment was repeated with other vaginal epithelial cultures with similar results.

activity-containing HPLC fraction, we determined the psoriasin content of HPLC fractions by ELISA (**Figure 5a,b**) and indeed found immunoreactivity co-eluting with the HPLC fractions containing the 11,366 Da protein. Individual vaginal fluids showed variable contents of HNP-1, lysozyme, and psoriasin as antimicrobial components with highest variability for HNP-1 and lysozyme (data not shown). Of 10 vaginal fluids

that we analyzed, 4 showed in addition to psoriasin, HNP-1, and lysozyme, an *E. coli*-cidal activity peak, which in some cases exceeded the antimicrobial activity originating from psoriasin (**Figure 5b**).

Preliminary biochemical analyses of these activity-containing HPLC fractions (sodium dodecyl sulfate-polyacrylamide gel electrophoresis-PAGE analyses, ESI-MS analyses, peptide mapping

Figure 4 Vaginal fluids contain high amounts of psoriasin. Psoriasin was quantified in vaginal lavages by enzyme-linked immunosorbent assay (ELISA) and western blotting analysis. **(a)** ELISA results for four different vaginal fluids (VFs) from healthy donors are shown. Psoriasin concentrations as high as $1.8 \mu\text{g ml}^{-1}$ were detectable in vaginal fluids. **(b)** Western blot analysis of two stratum corneum (SC) extracts and of four different VFs is shown. SC extract ($30 \mu\text{g}$) and VFs ($3 \mu\text{g}$) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). **(c)** The quantity of psoriasin in vaginal lavages was further analyzed by two-dimensional electrophoresis followed by western blot analysis. After blotting onto a polyvinylidene difluoride (PVDF) membrane, total proteins were stained using deep purple (red spots, left panel). The same membrane was further incubated with a mouse monoclonal antibody raised against human psoriasin. A single spot showing an isoelectric point of 5.6 and a molecular mass of 11.3 kDa was detected (green spot, right panel). The right panel shows an overlay of the total protein stain and the western blot analysis. One representative experiment of three is shown.

experiments, and tandem mass spectrometry sequencing experiments) did not reveal any conclusive data, which suggests that this activity does not originate from a protein, but from a low molecular mass substance of yet unknown structure.

DISCUSSION

Because of its anatomical location, the female urogenital tract is constantly exposed to a variety of pathogens such as *Candida* as well as Gram-positive and Gram-negative bacteria, protozoa, and viruses. *E. coli* is one of the major potential bacterial pathogens in this location, and although this bacterium is a frequent cause of urinary infections,^{16,17} it is rarely able to establish infections of the genital tract. This suggests that potent defense mechanisms exist within the outer female reproductive tract to prevent infection by *E. coli*.

In this study we show that vaginal fluids of healthy women indeed show strong bactericidal activity against *E. coli* and that psoriasin, accounting for up to 3% of the total protein content of vaginal fluids, is an important part of the *E. coli*-cidal defense in this environment. In skin, the patchy expression pattern of psoriasin correlates with bacterial load, suggesting a local induction of psoriasin by bacterial components.^{13,15} In contrast to observations of normal skin,¹³ normal vaginal and ectocervical

epithelium show strong constitutive psoriasin expression. This may be a consequence of continuous exposure to fecal bacteria or bacterial components. However, our additional observation that *in vitro* reconstructed vaginal epithelium, grown in the presence of antibiotics, expresses and secretes (up to 10 ng ml^{-1}) high levels of psoriasin suggests that even in the absence of bacteria vaginal epithelium has the capacity to produce psoriasin. Preformed psoriasin could thus prevent *E. coli* invasion, but if this failed, could be upregulated to combat increased numbers of bacteria. In this context it is interesting that we regularly observed dispersed cells within the suprabasal layers of vaginal epithelium that were even more strongly positive for psoriasin than the surrounding cells. A similar phenomenon was also observed in the *in vitro* reconstructed organotypic vaginal model. It is tempting to speculate that these cells might represent storage spaces able to release preformed psoriasin in case of injuries and disruption of the vaginal epithelium.

The expression pattern of psoriasin in vaginal epithelium shows some similarities to its expression in tongue mucosa.¹⁸ However, in contrast to the tongue mucosa, in which the basal as well as the most superficial epithelial cell layers showed no psoriasin expression, in the vagina all suprabasal cell layers were strongly positive for psoriasin. The presence of

Figure 5 Psoriasin contributes to *Escherichia coli*-cidal activity of vaginal fluid. **(a)** A vaginal lavage of a healthy donor was separated by micropore, wide-pore C2C18 reversed-phase high performance liquid chromatography (RP-HPLC). Bound proteins were eluted using a linear gradient of increasing acetonitrile concentrations and recorded at 215 nm. The resulting chromatogram is shown in the upper panel. The amount of psoriasin in HPLC fractions was determined by a psoriasin-specific enzyme-linked immunosorbent assay (ELISA; middle panel). Aliquots (2 μ l) of each fraction were tested for their bactericidal activity against *E. coli* using the radial diffusion assay system. The diameter of the clearing zone of each fraction is indicated in the lower panel. Masses detected upon electrospray ionization mass spectrometry (ESI-MS) analyses of selected fractions are indicated. The following proteins have been identified by ESI-MS: human neutrophil peptide-1 (HNP-1, 3.442 Da), lysozyme (14.692 Da), psoriasin (11.366 Da), and S100A8 (10.834). A representative analysis is shown. **(b)** A vaginal lavage of another healthy donor was separated as described in **a**. Note the presence of an additional peak of antimicrobial activity eluting between HNP-1 and psoriasin. Although ESI-MS analyses identified HNP-1 (3.442 Da), lysozyme (14.692 Da), and psoriasin (11.366 Da), the unknown antimicrobial compound did not show any signal > 1.000 Da. A representative chromatogram, which shows the highest amounts of the unknown antibiotic, is shown. This peak was also present in three additional vaginal lavages, but with lower intensity (data not shown).

sometimes excessive amounts of psoriasin in tongue-rinsing fluid may indicate permanent psoriasin release from the uppermost epithelial tongue layers. It is therefore conceivable that psoriasin is mainly retained in vaginal epithelium and

released upon yet unknown stimuli, whereas it is constitutively released from the tongue.

Our HPLC analyses of vaginal fluid from healthy women suggest that the psoriasin content is highest when two additional

AMPs, HNP-1 and lysozyme, are present (**Figure 5a,b**). The presence of HNP-1, an AMP derived from neutrophils, indicates that neutrophils have migrated into the vagina, possibly attracted by chemokines that were induced by bacterial components. Sporadic appearance of pathogens does not necessarily lead to clinically apparent infections, as overgrowth of these bacteria might be combated in an initial state by an enhanced release of AMPs. This finding suggests that, in addition to bacterial-derived factors such as flagellin,¹⁵ inflammatory cytokines involved in leukocyte trafficking might also contribute to the regulation of psoriasin in the female genital tract. Although regulation of psoriasin by inflammatory cytokines has been already shown for other cell types,^{15,19–21} a further more detailed study will be required to confirm that this assumption also holds true for the female genital tract. With respect to possible further regulators of psoriasin expression, it is conceivable that hormones such as estrogens could also have a role in the vagina as it has been shown previously for breast cancer cells.²²

We identified lysozyme as the second most abundant *E. coli*-cidal AMP in vaginal fluid, by its molecular mass and by mass mapping and tandem mass spectrometry sequencing (data not shown). Because both neutrophils and the vaginal epithelium produce lysozyme,²³ it is yet not clear which of these cells is the major source. Lysozyme is an exceptionally widespread bactericidal protein that is present in almost all body fluids. Its antimicrobial action occurs either by enzymatic or nonenzymatic pathways.²⁴ Although for Gram-positive bacteria its enzymatic activity is necessary for degradation of the cell wall leading to lysis and death,²⁴ the non-enzymatic action of lysozyme is characteristic for its activity against Gram-negative bacteria, such as *E. coli*.²⁴ The mechanism of its nonenzymatic activity is based on the insertion of this polycationic protein into the cell wall leading to the formation of ion channels.^{24–26} Recently, several studies implicated a synergistic or additive effect of other AMPs with lysozyme.^{27,28} Whether this also holds true for psoriasin is not yet known. The mode of action of psoriasin also seems to be nonenzymatic, as it depletes Zn^{2+} ions in a similar manner as the structurally related complex of S100A8 and S100A9 (calprotectin) that inhibit *Staphylococcus aureus* growth through chelation of the essential trace element Mn^{2+} .²⁹ Vaginal fluid has also been reported to contain calprotectin.³⁰ This complex is not stable during reversed-phase HPLC and dissociates. Indeed, our HPLC analyses of vaginal fluid sometimes revealed multiple peaks eluting at a higher acetonitrile concentration than psoriasin (**Figure 5a**), the main peak having a mass of 10.834 Da. This corresponds to S100A8, as shown by tandem mass spectrometry analyses (data not shown). However, no colicidal activities were detected in these fractions. Because neutrophils contain huge amounts of calprotectin and activated epithelial cells can produce only low amounts of it, the cellular source of calprotectin in vaginal fluid is also most likely the neutrophil.

In 4 of the 10 vaginal fluids that have been subjected to reversed-phase HPLC, we have observed an additional peak of antimicrobial activity. In some vaginal secretions (**Figure 5b**), this antimicrobial compound represented the most active antibiotic substance. Biochemical analyses (attempts to perform mass

mapping experiments and sequencing as well as molecular mass determination by ESI-MS) revealed that this compound is of low molecular mass. Although the use of antibiotics had been an exclusion criterion for the collection of vaginal fluid from healthy women, we cannot exclude with certainty an exogenous origin of the unknown substance. Further studies are necessary to analyze the identity and the source of this vaginal antibiotic at a molecular level.

In summary, we have shown that psoriasin is abundantly produced and released by vaginal epithelium, representing up to 3% of all proteins in the vaginal fluid. We suggest that by virtue of its high killing activity against *E. coli* and the fact that it is constitutively expressed, psoriasin represents an important component of the innate antimicrobial defense system of the female lower genital tract—although other, yet not characterized factor(s), seem to be also of relevance.

METHODS

Cell culture. Heat-inactivated *E. coli* bacteria were generated by incubating overnight at 37°C in LB medium. They were then washed and heat inactivated at 65°C for 30 min in phosphate-buffered saline. After centrifugation the supernatants were diluted 1:100 in maintenance medium (MatTek, Ashland, MA) and used for stimulation. *In vitro* organotypic vaginal cultures (EpiVaginal Tissue Model) were obtained from MatTek Corporation. The organotypic cultures were incubated in assay medium (MatTek Corporation) with or without supernatants of *E. coli* cultures as described previously.¹⁵ After 24 h the epithelium was collected for immunohistochemistry and western blot analysis.

Recovery of vaginal lavages and stratum corneum extract. Vaginal lavages were obtained from healthy women after obtaining informed consent. All vaginal lavages analyzed were obtained from women between 18 and 75 years of age. They had no acute sexually transmitted infections currently or in the past 2 years and showed no clinically apparent signs of vaginal infection. The stage of the menstrual cycle was not determined. The women were not treated with antibiotics. Vaginas were rinsed with 2 ml physiological NaCl solution that was immediately recollected with a sterile syringe. A contamination of the syringe with the skin was avoided by the gynecologist. These vaginal lavages were passed through a 45- μ m sterile filter to remove cellular elements. After high-speed centrifugation the fluids were aliquotted and stored at -80°C for further use. Stratum corneum extracts were prepared by incubation of human skin scales with physiological NaCl solution.

Tissue samples and immunostaining. Paraffin-embedded samples of vagina ($n = 10$), cervix ($n = 10$), vulva ($n = 5$), and normal healthy skin ($n = 10$) were analyzed. Healthy skin samples were obtained from mammary reduction plastic surgery from the Department of Plastic Surgery. All other specimens were tissue bank samples that had been obtained for diagnostic purposes by the Department of Pathology. The samples were tumor free. Immunohistochemical staining was performed on 5- μ m thick sections of formalin-fixed, paraffin-embedded tissues. After deparaffinization and hydration, sections were pre-treated with microwave in citrate buffer (Dako, Vienna, Austria) and stained with a monoclonal anti-psoriasin antibody ($1\ \mu\text{g ml}^{-1}$; Abcam, Cambridge, UK) using fast red as substrate (Labvision, Fremont, CA). All experimental procedures were conducted in compliance with the Declaration of Helsinki Principles.

Western blot analysis. Western blot analysis was performed as described previously,³¹ with minor modifications. Organotypic vaginal epithelium was lysed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer. After sonication and centrifugation, proteins were size

fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis through an 8–18% gradient gel (Amersham Pharmacia Biotech, Uppsala, Sweden) and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Immunodetection was performed with a mouse anti-psoriasin antibody ($1 \mu\text{g ml}^{-1}$; Abcam), followed by a horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G antiserum (dilution 1:10,000; Amersham). Reaction products were detected by chemiluminescence with the ChemoGlow reagent (Biozyme Laboratories, South Wales, UK) according to the manufacturer's instructions.

Psoriasin ELISA. Vaginal lavages were analyzed by ELISA as described previously.¹³

Two-dimensional electrophoresis. Samples for two-dimensional electrophoresis were dialyzed against double-distilled water for 4 h at 4 °C. Immobiline Dry Strips pH 3–10 (GE Healthcare) were rehydrated overnight in rehydration buffer (8 M urea, 0.5% (w/v) CHAPS, 0.2% (w/v) dithiothreitol, 0.5% (v/v) Phormalyte (GE Healthcare) pH 3–10, and 0.002% bromophenol blue) including 20 μl of dialyzed sample. Isoelectric focusing was performed using a Multiphor II Electrophoresis Unit (GE Healthcare). Strips were equilibrated in 2% sodium dodecyl sulfate, 50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, and 0.002% bromophenol blue according to the Ettan DIGE User's Manual, (GE Healthcare, 18-1173-17 Edition AA (2002)) and then laid on 20×26 cm Ettan DALT precast gels that were run in an Ettan DALTsix electrophoresis unit (GE Healthcare) under denaturing conditions. After electrophoresis, the gels were blotted to Hybond-LFP (low-fluorescent polyvinylidene difluoride) membranes for 20 h at 200 V using a NovaBlot Multiphor II system (GE Healthcare). The protein blots were stained with Deep Purple Total Protein Stain according to the manufacturer's protocol (GE Healthcare), and then scanned with the Ettan DIGE Imager. Images were analyzed using ImageQuant TL (GE Healthcare) and Melanie software (Genebio). After total protein staining, the blots were incubated in ECL advance blocking solution (GE Healthcare) at room temperature for 2 h. The blots were then incubated with the monoclonal mouse anti-psoriasin primary antibody ($1 \mu\text{g ml}^{-1}$ in ECL advance blocking solution) overnight at 4 °C. They were washed twice quickly, then twice for 5 min each in phosphate-buffered saline + 0.1% Tween-20, and then incubated for 1.5 h with the secondary antibody (ECLPlex goat- α -mouse IgG-Cy5; 1:2,500; GE Healthcare). The membranes were then washed three times quickly, then four times for 5 min each in phosphate-buffered saline + 0.1% Tween-20 followed by two brief washes in phosphate-buffered saline before scanning on the Ettan DIGE Imager. Images were analyzed using ImageQuant TL (GE Healthcare) and Melanie software (Genebio).

Antimicrobial assay. The antimicrobial activity of vaginal fluids was estimated with a microdilution assay system described previously.¹³ Test organisms (*E. coli*, ATCC 35218) were incubated for 3 h at 37 °C in physiological NaCl solution, containing 1% (v/v) trypticase soy broth. The antibiotic activity was analyzed by plating of serial dilutions of the incubation mixtures on LB-agar plates and determination of the number of colony-forming units the next day. Antimicrobial activity in HPLC fractions was analyzed using the radial-diffusion assay system as described previously.³² In all, 2 μl aliquots of each fraction were tested for their bactericidal activity against *E. coli*. The diameter of the clearing zone of each fraction after 24 h incubation at 37 °C was measured.

High performance liquid chromatography. Individual vaginal fluids of healthy donors were separated by micropore, wide-pore C2C18 reversed-phase HPLC using the Smart-system, a micropore analytical column and manual collection of HPLC fractions. Proteins were eluted with a gradient of increasing concentrations of acetonitrile in 0.1% (v/v) trifluoroacetic acid (flow rate, 100 $\mu\text{l min}^{-1}$). Aliquots (2 μl) of each fraction were lyophilized, dissolved in 5 μl of 0.01% (v/v) aqueous acetic acid, and

tested for antimicrobial activity against *E. coli* (ATCC 11303) by a radial diffusion plate assay,³² and for psoriasin by a specific ELISA.

ESI-MS analyses. Protein and peptide mass determinations were performed by ESI-MS analyses using a Quadrupole time-of-flight hybrid mass spectrometer (Q-TOF II; Waters Micromass, Milford, MA) operated in positive ionization mode.

ACKNOWLEDGMENTS

We thank Ms Heidemarie Rossiter for critical reading of the paper, C. Martensen-Kerl, J. Quitzau, and H. Hinrichs for excellent technical assistance, and Joachim Bartels for ESI-MS analyses. Part of this study has been supported by Deutsche Forschungsgemeinschaft (SCHR 305/5-1).

DISCLOSURE

The authors declared no conflict of interest.

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